Lactic acid bacteria fermentations in oat-based suspensions

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2002

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Food Science and Technology/LWT (2000) 33, 525-530.

PAPER I

Lebensm.-Wiss. u.-Technol., 33, 525–530 (2000)

Lactic Acid Bacteria in an Oat-based Non-dairy Milk Substitute: Fermentation Characteristics and Exopolysaccharide Formation

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(Received May 9, 2000; accepted July 20, 2000)

Nine strains of lactic acid bacteria were tested for fermentation characteristics in an oat- based, non-dairy milk substitute, Mill Milk™ (MM medium). Viable counts, aroma formation, lactic acid production and viscosity were the parameters investigated. In general, bacterial strains grown at 30 °C yielded a better flavour and higher viable counts than those incubated at 37 °C. Strains were selected for their capacity to produce exopolysaccharides in a semi-defined medium. Lactobacillus delbrueckii *subsp.* bulgaricus *NCFB 2772 was chosen for further fermentation experiments, using yoghurt as a control. Production of exopolysaccharide and shear thinning properties were measured using a viscosimetric method. A combination of temperature stress and an increased carbon/nitrogen ratio was shown to affect the production of exopolysaccharide. Increased viscosity was found after incubation at a low temperature using glucose as a supplementary carbon source. The production of exopolysaccharides was also favoured by prolonged incubation times. The use of physical factors such as time and temperature in combination with chemical factors, i.e. media and carbon/nitrogen levels, were crucial in the course of improving exopolysaccharide production during fermentation in the MM medium.*

Keywords: Non-dairy; *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772; Mill Milk™; EPS

Introduction

During recent years there has been an increased demand from consumers for non-dairy milk substitutes with high acceptance and functionality. The commercial, non-dairy milk substitute, Mill Milk™ (MM medium) is a 'whole-product' entirely derived from oats. It is based on a unique, patented enzymatic process (U.S. patent No. 5686123) which involves dry milling or wet milling of rolled oats or oat flour at 60 °C, followed by an enzymatic reaction using β-amylase. Maltose and β-limit dextrins are thus the main carbohydrates in the final product formed from the oat starch. After the enzymatic procedure the insoluble fibres can optionally be separated using a decanting step. The final product, high or low in insoluble fibres, can be modified further by the addition of nutrients, oil and flavourings (1). Previous studies have shown that this beverage has high acceptability concerning taste and flavour and also exhibits cholesterol and lipid-lowering effects (2, 3).

In recent years the focus on lactic acid bacteria producing exopolysaccharides (EPS) has been on their ability to enhance and improve texture and viscosity (4, 5). Yoghurts with EPS-producing bacterial strains show less shear thinning behaviour in comparison with yoghurt made with strains not producing EPS (6). This structural property would give rise to a new generation of *in situ* produced thickeners. This is of general interest, as there is an increasing demand from manufactures to decrease the addition of stabilizers in yoghurt products (7).

Most investigators have studied the production of exopolysaccharides in milk-based media. To date very few studies on exopolysaccharide-producing strains, grown in non-dairy, fermented products have been reported (8). Moreover, to our knowledge no information has been published concerning exopolysaccharide production by lactic acid bacteria in an oat-based medium.

The objective of this paper was to investigate the possibilities of using the Mill Milk™ (MM medium) to develop new applications of fermented vegetarian alternatives to products based on cow's milk. Nine strains of lactic acid bacteria were selected for the fermentation of the

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0023-6438/00/080525 + 06 $35.00/0
doi:10.1006/fstl.2000.0718

MM medium. The change in viscosity after fermentation, using an exopolysaccharide-producing bacterial strain, was taken as a measure for the capacity of the strain to produce exopolysaccharides in the MM medium.

Materials and Methods

Bacterial strains and media

In total, nine mesophilic bacterial strains with optimal growth temperatures at 30 °C or 37 °C were used. *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 and *Leuconostoc dextranicum* NCFB 2706 were obtained from The National Culture Collection of Food Bacteria, Aberdeen, Scotland. *Lactobacillus brevis* DSMZ 1269, *L. kefiri* DSM 20485 and *L. delbrueckii* subsp. *bulgaricus* DSM 20081, *Streptococcus thermophilus* DSM 20259, *Pediococcus damnosus* DSM 20331, *Propionibacterium propioniacidici* DSM 20272 and *Leuconostoc mesenteroides* DSM 20240 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. *Propionibacterium propioniacidici* DSM 20272 was cultured before use in propionibacterium broth (DSM-medium 91) composed of 10 g/L casein peptone, tryptic digest (Difco, Detroit, Michigan, U.S.A.), 5 g/L yeast extract (Difco), 10 g/L Na-lactate (Merck). *Streptococcus thermophilus* DSM 20259 was cultured in corynebacterium broth (DSM-medium 53) composed of 10 g/L casein peptone, tryptic digest (Difco), 5 g/L yeast extract (Difco), 5 g/L sodium chloride, 5 g/L glucose (Merck). All other bacterial strains were cultured in MRS broth (Merck, Darmstadt, Germany) (9). To stock cultures 20% (v/v) of glycerol was added to the media and the cultures were kept at −80 °C. Before use the bacterial strains were propagated twice in respectively growth medium supplemented with cysteine-HCl 0.05% (v/v) (Fluka, Buchs, Switzerland) at 30 or 37 °C. The transfer inoculum was a 1% (v/v) taken from a culture grown for 24 h in a fresh medium under anaerobic conditions using anaerobic jars (85% N_2, 10% H_2, 5% CO_2). After 24 h of growth the cell concentration had reached approximately 1×10^8 cfu/mL for all bacterial strains tested. The solid media were prepared by the addition of 15 g/L of granulated agar (Difco) to the broths. The exopolysaccharide selective medium (ESM medium) (10) contained (g/L) skimmed milk powder (SMP) (Oxoid, Basingstoke, U.K.), 90, yeast extract (Difco), 3.5, peptone (Difco), 3.5, and glucose (Merck), 10. Mill Milk™ (MM medium), was kindly provided, aseptically packed, by Ceba AB, Sweden, and analysed for protein, fat, different carbohydrates, dietary fibers, various vitamins and minerals by an authorized laboratory (AnalyCen Nordic AB, Kristianstad, Sweden). The results are shown in **Table 1**. Control yoghurt was obtained from Skånemejerier, Sweden. Glucose, fructose or sucrose (Merck) were added to 5% (w/v) to the MM medium, to enhance the production of exopolysaccharides.

The screening procedure

The nine strains were screened for their capacity to ferment MM medium. The fermentations were set up in sterile and sealed bottles containing 200 mL of MM inoculated with a 24 h culture at an inoculum concentration of 5% (v/v). The bottles were incubated at 30 or 37 °C for 24 h. At the end of this incubation, plates of either MRS agar (Merck), propionibacterium agar or corynebacterium agar were incubated for viable counts at 30 or 37 °C for 72 h. The ESM medium, supplemented with 0.05% (v/v) cysteine-HCl, was inoculated with a 5% (v/v) inoculum of a 24 h culture. Results were expressed as colony forming units (cfu/mL). Lactic acid was determined as titratable acidity expressed as percentage according to (11). The flavour of the sample was evaluated by smelling and tasting the sample after incubation. Flavour was judged according to a scale, low (+), medium (+ +) and high (+ + +). Yoghurt was used as a control.

The selection of an exopolysaccharide bacterial strain using the ESM medium was made by visual inspection of the ropiness of the medium. The culture was defined as ropy after incubation at 30 or 37 °C for 24 h when a thread of at least 10 mm was formed when a loop of the culture is lifted from the surface of the broth.

Table 1 Chemical composition of Mill Milk™ (MM medium)

Component	MM medium (g/100 g)
Protein (g)	1.1
Fat (g)	1.5
Maltose (g)	4.2
Maltodextrin (g)	2.7
Dry matter (%)	11
Total fibre (g)	0.8
β-glucan (g)	0.4
α-tocopherol (mg)	0.1
Thiamin (mg)	0.04
Riboflavin (μg)	9.6
Niacin (mg)	0.1
Folic acid (μg)	3.3
Pyridoxine (mg)	0.01
Iron (mg)	0.1
Magnesium (mg)	4.7
Manganese (mg)	0.1
Phosphorus (mg)	27
Sodium (mg)	11
Zinc (mg)	0.1

Viscosity measurement

MM medium inoculated with the different strains was incubated at four different temperatures, 25, 30, 37 and 45 °C for 24 h. Samples were also incubated at 25 and 30 °C for 72 h. Glucose, fructose or sucrose supplements were added to the medium as sterile solutions before incubation. After the incubation, and subsequent cooling, to 25 °C the viscosity was measured on 150 mL samples. This measurement was performed using a Bohlin Visco 88 (Bohlin Reologi AB, Lund, Sweden) with the spindle C30 at a shear rate of 1207 s^{-1} for 120 s and expressed as mPas. The samples were then stored at 6 °C for 24 h to regain viscosity, where-after viscosity measurements were

again performed at 25 °C. As a control, for comparing viscosities and decrease in pH due to chemical and microbial acidification, the samples were chemically acidified by addition of 2% (w/v) glucono-δ-lactone (GDL) (Sigma Chemical Co) (12) to the MM medium before incubation. All experiments were performed in triplicate and the results are the arithmetic mean ± SD.

Results

Fermentation of MM medium with lactic acid bacteria

Nine strains of lactic acid bacteria were examined with respect to their growth and formation of metabolites in MM medium (**Table 2**). Six of the strains tested gave a final pH of 4.5 or lower after 24 h of incubation at 30 or 37 °C. Acidification of the medium by GDL gave a final pH of 2.9. Eight of the strains gave a viable count higher than 10^7 cfu/mL. One strain, *Leuc. mesenteroides* DSM 20240, developed a pleasant flavour and a good taste when grown for 24 h in the MM medium. Two strains, *Leuc. dextranicum* NCFB 2706 and *L. kefiri* DSM 20485, formed an acceptable flavour. The other strains tested produced a flavour of low acceptance.

Screening the lactic acid bacteria for exopolysaccharide production using a semi-defined medium (ESM medium)

Five of the nine strains used in this study exhibited some ropiness in the ESM medium after 24 h of incubation (**Table 2**). The *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 strain yielded the highest ropiness and was selected for further fermentation experiments to quantify the production of exopolysaccharides in the MM medium using a viscosimetric method.

Viscosity in the MM medium after incubation with L. delbrueckii *subsp.* bulgaricus *NCFB 2772 using different carbon sources*

An increase in viscosity after incubation was observed in all the samples tested (**Table 3**). The initial viscosity of a sample with no supplementation of any carbon source was highest at the incubation temperature of 25 °C. MM samples supplemented with glucose exhibited a higher initial viscosity compared to the yoghurt control except for those samples incubated at 45 °C. The shear thinning behaviour was more obvious for the MM samples than in the yoghurt control. MM sample supplemented with glucose and incubated at 30 °C showed the highest final

Table 2 Fermentation characteristics of lactic acid bacteria in MM medium

Bacterial strain	Cell number (cfu/mL)	Final pH‡	Lactic acid (%)	Flavour*	Growth temperature (°C)	EPS†
Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2772	1.0×10^7	4.5	0.22	+	37	+
Lactobacillus brevis DSM 1269	1.2×10^8	4.5	0.22	+	30	−
Lactobacillus kefiri DSM 20485	3.2×10^8	4.0	0.37	++	30	−
Lactobacillus delbrueckii subsp. *bulgaricus* DSM 20081	5.0×10^7	5.1	0.14	+	37	+
Streptococcus thermophilus DSM 20259	2.0×10^7	4.7	0.18	+	37	+
Pediococcus damnosus DSM 20331	1.3×10^8	4.0	0.37	+	30	−
Propionibacterium propioniacidici DSM 20272	4.0×10^7	4.9	0.15	+	30	−
Leuconostoc mesenteroides DSM 20240	2.0×10^7	3.9	0.41	+++	30	+
Leuconostoc dextranicum NCFB 2706	5.0×10^7	3.6	0.44	++	30	+

* Subjective judgement of the fermented samples for pleasant flavour. (+) low flavour production; (++) medium flavour production; (+++) high flavour production.
† Screening procedure was performed using an exopolysaccharide selecting medium (ESM).
‡ Initial pH in MM medium was 7.1.

Table 3 Viscosity* of MM medium after 24 h of incubation with *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 at four different temperatures using different carbon sources

Incubation temperature (°C)	Time (s)	MM		MM + glucose		MM + sucrose		MM + fructose		MM + GDL†		MM control		Yoghurt control	
		0	120	0	120	0	120	0	120	0	120	0	120	0	120
25	Viscosity (mPas)	39 ± 5	22 ± 2	58 ± 11	28 ± 5	39 ± 7	21 ± 2	28 ± 4	20 ± 1	11 ± 0	11 ± 0	15 ± 2	15 ± 1	60 ± 1	43 ± 2
30		36 ± 5	21 ± 1	77 ± 13	36 ± 5	44 ± 1	29 ± 1	25 ± 2	20 ± 1	11 ± 0	11 ± 0				
37		31 ± 3	20 ± 3	65 ± 16	34 ± 6	38 ± 0	25 ± 1	24 ± 0	19 ± 0	11 ± 1	11 ± 0				
45		27 ± 3	19 ± 1	41 ± 10	24 ± 4	34 ± 1	21 ± 0	23 ± 1	19 ± 0	10 ± 0	10 ± 0				

* Data are the means ± SD of three measurements.
† Chemically acidified MM medium using glucono-δ-lactone.

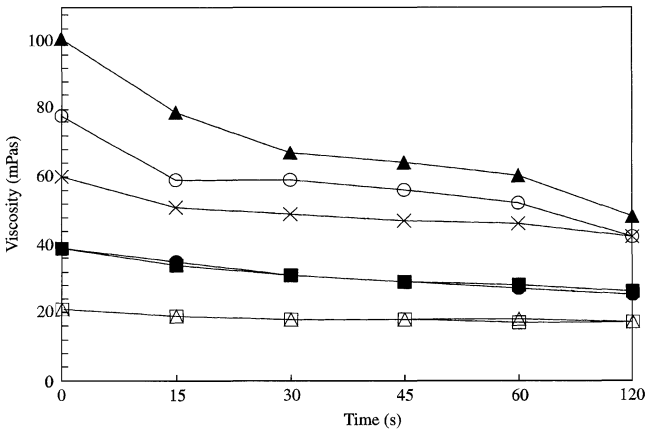

Fig. 1 Viscosity measurements during 120 s of shear stress in the MM medium after 72 h of incubation with *L. delbrueckii* subsp. *bulgaricus* NCFB 2772. The medium was supplemented with three different sources of carbon at two different incubation temperatures. (●), MM medium + sucrose at 25 °C; (■), MM medium + sucrose at 30 °C; (▲), MM medium + glucose at 25 °C; (○), MM medium + glucose at 30 °C; (□), MM medium + fructose at 25 °C; (△), MM medium + fructose at 30 °C; (×), yoghurt control

Table 4 Recovery* of viscosity† in MM medium supplemented with glucose and fermented with *L. delbrueckii* subsp. *bulgaricus* NCFB 2772. Intial viscosity was measured after 24 hours of incubation at 30 °C

Incubation time (h)	Initial viscosity (mPas)	‡Viscosity after storage (mPas)	Recovery (%)
24	36 ± 5	27 ± 3	75 ± 2
72	48 ± 4	32 ± 2	67 ± 3
Yoghurt (control)	43 ± 2	43 ± 2	100 ± 0

* Recovery was calculated as the difference between the initial viscosity and the viscosity after storage.
† Data are the means ± SD of three measurements.
‡ Viscosity is measured after 24 h of storage at 6 °C.

viscosity of all MM samples tested. As comparison, the highest viscosity for the sucrose-supplemented sample was also observed at 30 °C. Using fructose as a carbon source only a small increase in viscosity was found after incubation. No change in viscosity was observed in the chemically acidified samples.

Combination of long incubation time and low temperature using three different carbon sources for exopolysaccharide production of L. delbrueckii *subsp.* bulgaricus *NCFB 2772 in MM medium*

The effect of the addition of different carbon sources on viscosity is shown in **Fig. 1**. The sample supplemented with glucose incubated at 25 °C, had higher final viscosity compared to yoghurt control and the other samples tested. The glucose-supplemented sample incubated at 30 °C had a final viscosity similar to that of the yoghurt control. Incubation at 25 °C for 72 h gave a higher final viscosity compared to a 24 h incubation for the glucose-supplemented sample (**Fig. 1**). The samples supplemented with fructose did not exhibit any change in viscosity after incubation at either temperature.

Recovery of viscosity

The viscosity measurements were repeated after 24 h for MM samples supplemented with glucose and incubated at 30 °C for 24 and 72 hours. The difference in viscosity before and after storage is shown in **Table 4**. It was observed that the percentage of recovery was lower for the samples of MM medium in comparison to the control.

Discussion

The evaluation of suitable lactic acid bacteria to ferment a non-dairy food product is a crucial step in the development of new products within the non-dairy field. In this present work we show the capacity of nine lactic acid bacteria to ferment a non-dairy product, Mill Milk™ (MM medium), as well as, exopolysaccharide (EPS) formation by slime-producing strains. The viable counts and pH reduction were comparable to those in a medium based on cow milk (13). This shows that the MM medium contains components suitable for fermentation by lactic acid bacteria. *Leuc. mesenteriodes*, *Leuc. dextranicum*, *P. damnosus* and *L. kefiri* exhibited higher lactic acid production during fermentation than the other bacterial strains. The samples fermented with these four strains had a pleasant flavour. Samples fermented with the well-documented slime producer *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 did not seem to exhibit phase separation to the same extent as the other fermented samples. Thus homogenous samples were more likely to be formed after incubation with this bacterial strain.

A simple screening method for the capacity of producing EPS was used. Five of the nine strains tested showed some ropiness in the liquid medium. *Lactobacillus delbrueckii* subsp. *bulgaricus NCFB 2772* exhibited the highest slime production in both the semi-defined medium (ESM) and the MM medium (data not shown) and was therefore selected for further experiments to investigate the ability to improve viscosity in the MM medium. Maximal EPS production in the MM samples supplemented with glucose took place at 30 °C when using an incubation time of 24 h. When the incubation time was prolonged to 72 h, the optimal temperature for EPS production was 25 °C. This shows that the production of EPS does not occur at the optimal temperature for growth but is more likely at lower temperatures, which is in accordance with earlier findings (13). On the other hand, one study has reported enhanced EPS production at high incubation temperatures (14). After shear stress, the final viscosity was still higher in the MM medium supplemented with glucose than in the yoghurt control. The increase in viscosity with the addition of glucose is in accordance with other studies (15). A change in viscosity after incubation in the presence of sucrose was also observed. Using the same shear stress treatment after recovery, it was shown that the capacity to regain viscosity was similar to that in a study using ropy bacterial strains in a milk-based medium (16). Other groups (5, 17) have reported difficulties in using viscosity as a parameter for the production of EPS. One of the reasons might be due to alterations in the protein components as well as the production of fermentation metabolites, such as lactic acid, which interfere with the viscosity measurements during long incubation times. The final pH in the chemically acidified samples was also much lower than that in the fermented samples. No effect on the viscosity was observed after incubation with the GDL-supplemented sample. Contrary to our results obtained with MM medium, GDL-acidified milk-based samples have been reported to have both a higher final viscosity and pH when the same amount of GDL was used (18). That a final pH as low as 2.9 did not increase the viscosity in the MM medium is mainly due to the low protein content in the medium. These proteins are obviously not likely to coagulate during chemical changes caused by fermentation metabolites or by the addition of GDL to an extent, which would affect the final viscosity. The same effect was observed when the MM medium was fermented with bacterial strains that did not exhibit any EPS formation in the medium. The final pH values of these fermentations were all between 3.6–5.1. This shows that it is suitable to use viscosity measurements for the investigation of exopolysaccharide production in the MM medium. The study also shows that the MM medium possess a lower buffering capacity in comparison to milk-based media. A deeper understanding of the function of EPS-producing lactic acid bacteria in the role of improving the texture, consistency and sensory properties in media other than milk is important for in the long-term goal of developing, new functional, fermented, non-dairy milk substitutes. Further studies should also focus on possibilities to improve texture in MM medium supplemented with different carbon sources and to investigate other strains or combinations of strains from lactic acid bacteria. Our study shows that the Mill Milk™ (MM medium) is a suitable medium for lactic acid bacteria fermentation.

Acknowledgements

SL-foundation (Skånska Lantmännen, Sweden) and Ceba AB financially supported the research presented in this study.

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